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Tumor Suppressor *miR-22* Determines p53-Dependent Cellular Fate through Post-transcriptional Regulation of p21

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Abstract

Selective activation of p53 target genes in response to various cellular stresses is a critical step in determining the ability to induce cell-cycle arrest or apoptosis. Here we report the identification of the microRNA *miR-22* as a p53 target gene that selectively determines the induction of p53-dependent apoptosis by repressing p21. Combinatorial analyses of the AGO2 immunocomplex and gene expression profiles identified *p21* as a direct target of *miR-22*. Induction of p21 was inhibited by *miR-22* after exposure to the genotoxic agent Adriamycin (doxorubicin; Bedford Laboratories), sensitizing cells to p53-dependent apoptosis. Interestingly, the activation of *miR-22* depended on the intensity of the stresses that induced cells to undergo apoptosis in the presence of *p21* suppression. Our findings define an intrinsic molecular switch that determines p53-dependent cellular fate through post-transcriptional regulation of p21. *Cancer Res*; 71(13); 4628–39. ©2011 AACR.

Introduction

The p53 tumor suppressor network plays a crucial role in the prevention of malignant transformation in normal cells by maintaining the integrity of signaling pathways in response to various oncogenic stresses, including DNA damage, acute activation of oncogenes, and hypoxic conditions (1). The outcome of p53 activation in response to cellular stresses ranges from the induction of cell-cycle arrest for DNA repair to apoptosis for the complete elimination of damaged cells (2–4). The commitment to one of these alternative cellular fates depends on the set of p53 target genes induced by different stresses. Induction of cell-cycle arrest is mediated by the activation of the cyclin-dependent kinase inhibitor *CDKN1A* (hereafter referred to as p21), whereas apoptosis is induced by the activation of pro-apoptotic genes, including *NOXA* (5), *PUMA* (6), and *BAX* (7) that encode the regulators of intrinsic apoptosis pathways.

Post-translational modifications of p53 are involved in the selective activation of its various target genes leading to apoptosis (8, 9). Phosphorylation of p53 at serine 46 (Ser46), mediated by HIPK2 (10), regulates apoptotic pathways through the activation of *p53AIP1* (11). Furthermore, acetylation of p53 at lysine 120 (K120) by Tip60 is essential for the expression of *PUMA* (12). Ongoing work focuses on the elucidation of p53 function and its regulation as a transcriptional factor.

Recently, the regulation of gene expression by small noncoding RNAs, including microRNAs (miRNA), has been reported to play crucial roles in the maintenance of homeostasis in a wide range of cellular processes, including differentiation, control of cell proliferation, and stress responses (13–15). The important feature of miRNAs is the targeting of multiple cellular mRNAs, resulting in the efficient activation or repression of intracellular or intercellular signaling networks at specific times during animal development. miRNA dysfunction therefore causes defects in the integration of signaling networks essential for the maintenance of cellular homeostasis.

miRNA dysfunction has been suggested as a dominant cause of the onset of human disorders, especially cancers. Indeed, aberrant expression of miRNA genes was observed in almost all types of human cancers (16, 17). As a consequence of miRNA dysfunction, cancer cells acquire properties that favor the activation of oncogenic pathways or the repression of tumor-suppressive networks, contributing to cancer progression and metastasis (18–22). *Mir-21* was shown to repress *PTEN*, activating the phosphoinositide 3-kinase (PI3K)–AKT pathway and reflecting its oncogenic role (23). By contrast, *miR-34a* was identified as a p53-regulated tumor-suppressive miRNA in human colon cancer and shown to induce p53-dependent apoptosis or premature senescence, forming a positive feedback loop with p53 (24–28). The function of miRNAs as oncogenes or tumor suppressor genes is therefore well known, and

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it implies that the incorporation of miRNA species as critical components of intracellular signaling pathways is crucial for the reconstitution of integrated cancer-related networks necessary to fully clarify the molecular basis of carcinogenesis.

To analyze the connection between miRNAs and signaling networks, a functional genetic screening method named "dropout assay" was recently established using a lentivirus miRNA expression library and a home-made microarray to quickly and efficiently isolate tumor-suppressive miRNAs (29). In the present study, an *in vitro* functional genetic screen and comprehensive genomic screens of clinical samples were used to identify tumor suppressor miRNAs in colon carcinogenesis, with the resulting identification of *miR-22* as a tumor suppressor gene. A p53–*miR-22*–p21 axis was identified as a crucial regulatory component involved in the determination of p53-dependent apoptosis. Our results suggest that *miR-22* is an intrinsic molecular switch or sensor for the determination of p53-dependent cellular fate in response to distinct stresses, and *miR-22* dysfunction could affect the anticancer barrier against various oncogenic insults.

Materials and Methods

Cell culture

HCT 116 (HCT 116 p53^{+/+}) and HCT 116 p53^{-/-} (30) were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD). These cell lines were authenticated by morphologic inspection, and mycoplasma testing using PCR. The activation of p53 pathways was confirmed by checking the induction of p53 target genes after exposure to DNA damage before starting the experiments. The SW480 colon cancer cell line was obtained from the American Type Culture Collection and authenticated as described above. Mutation of *TP53* was confirmed by sequencing. These cell lines were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% heat inactivated FBS in humidified air with 5% CO₂.

Clinical samples

Paired surgical specimens of primary human colon cancers and surrounding noncancerous colon tissue counterparts were obtained from patients treated at the Teikyo University Hospital (Mizonokuchi, Kanagawa, Japan) with documented informed consent in each case. Institutional review board approval for the analysis of clinical samples was obtained at each institute.

Functional miRNA dropout screening

Functional dropout screening to identify tumor suppressor miRNAs was carried out according to our recent publication (29). HCT 116 cells were transduced with a pooled lentivirus miRNA expression library (SBI) at a multiplicity of infections (MOI) of 3. Cells were incubated in complete medium for 3 days (P1) and subjected to sequential passages every 3 days. After 9 passages, genomic DNA was prepared from P1, P5, and P9 cells and subjected to array CGH analysis using a home-made microarray.

Quantitative real-time PCR

For quantitative expression analysis of miRNAs, total RNAs from colon cancer patients were reverse-transcribed by MultiScribe RT and miRNA-specific miRNA primers (ABI), and quantitative real-time PCR (qRT-PCR) was carried out by using a TaqMan microRNA assay kit (ABI). The comparative cycle threshold (C_t) method was applied to quantify the expression levels of miRNAs. Relative expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method. *U48* small nuclear RNA was used as an internal standard.

Chromatin immunoprecipitation sequencing

HCT 116 cells were treated with 5-fluorouracil (5-FU; 0.375 mmol/L) for 9 hours, and chromatin immunoprecipitation (ChIP) was conducted by using anti-p53, antimonomethylated or antitrimethylated histone H3 K4, or antitrimethylated histone H3 K36 antibodies. ChIP-isolated DNA was subjected to the sequencing using an Illumina platform.

AGO2-IP on ChIP analysis

The AGO2-IP on ChIP assay was carried out according to a previous report with minor modifications (31). In brief, HCT 116 cells stably expressing HA-AGO2 were transfected with either *miR-22* (Pre-miR precursor molecule, Ambion) or miR-NC (Pre-miR miRNA Precursor Molecules Negative Control #2, Ambion) for 24 hours, and immunoprecipitated using anti-HA agarose beads. AGO2-bound RNA was eluted in boiling water, and the Trizol-LS reagent was added to extract total RNAs. AGO2-bound total RNAs were cleaned further using an RNeasy column and subjected to microarray analysis.

Reporter plasmid construction and luciferase assay

Amplification of the 3' UTR of *p21* mRNA was carried out by PCR from HCT 116 genomic DNA using a primer set (Supplementary Table S1). The DNA fragment was fused to the 3' end of a *firefly* luciferase reporter gene in a pmirGLO dual luciferase vector (Promega). Site-directed mutagenesis of a *miR-22* target site of *p21* mRNA was carried out by using a PrimeSTAR Max high fidelity DNA polymerase using the pmirGLO-*p21* 3'UTR plasmid as a template. HCT 116 cells, seeded at 5×10^4 cells/mL, were cotransfected with 200 ng of reporter plasmid and 10 nmol/L of either *miR-22* or miR-NC using Lipofectamine 2000. After incubation for 24 hours, luciferase activities were determined by using a dual luciferase assay kit (Promega). Luciferase activity was normalized by *Renilla* luciferase activity as an internal standard.

Immunoblot analysis

Cells were lysed in lysis buffer consisting of 25 mmol/L Tris-HCl (pH7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.1% SDS and 1× proteinase inhibitor cocktail, and equal concentrations of protein samples were loaded on a 10% to 20% polyacrylamide gradient gel (ATTO). After electrophoresis, proteins were transferred to a PVDF membrane, and immunoblot analysis was conducted by the standard method.

Supplementary information

Supplementary information includes extended Materials and Methods, 8 figures, and 4 tables.

Results

Identification of *miR-22* as a candidate tumor suppressor miRNA by functional genetic and comprehensive genomic screens

A screening method for the efficient identification of tumor suppressor miRNAs in colon cancer was established and is depicted in Supplementary Fig. S1A. Tumor suppressor miRNAs were defined as follows; (i) repressor of cell proliferation, (ii) expression in normal colon tissue, (iii) high-frequency loss of their chromosomal positions, and (iv) downregulation in human colon cancers. Following these criteria, a functional genetic screening, namely a "dropout assay," was conducted using a lentivirus miRNA expression library (29) to isolate repressors of cell proliferation in a colon cancer cell line (Supplementary Fig. S1B). HCT 116 cells were transduced

with a pooled lentivirus library containing 454 miRNA species and propagated for 3 weeks with sequential passages. Genomic DNA from the first passage (P1), fifth passage (P5), and ninth passage (P9) cell populations was prepared, and copy numbers of each miRNA clone in these cells were compared by array CGH analysis using a home-made microarray (Supplementary Fig. S1B). A total 55 miRNA clones were reproducibly dropped out in a culture time-dependent manner (Supplementary Fig. S1C and Table S2). Among these dropout clones, 24 miRNAs were confirmed for their expression in normal tissue (Supplementary Fig. S1D). Furthermore, we carried out array CGH analysis (aCGH) to examine autosomal copy number aberrations using 24 colon cancer patients and finally identified 6 miRNA clones whose genes show hemizygous deletions in cancers with a high frequency (>30%), as candidates for tumor suppressor gene in colon

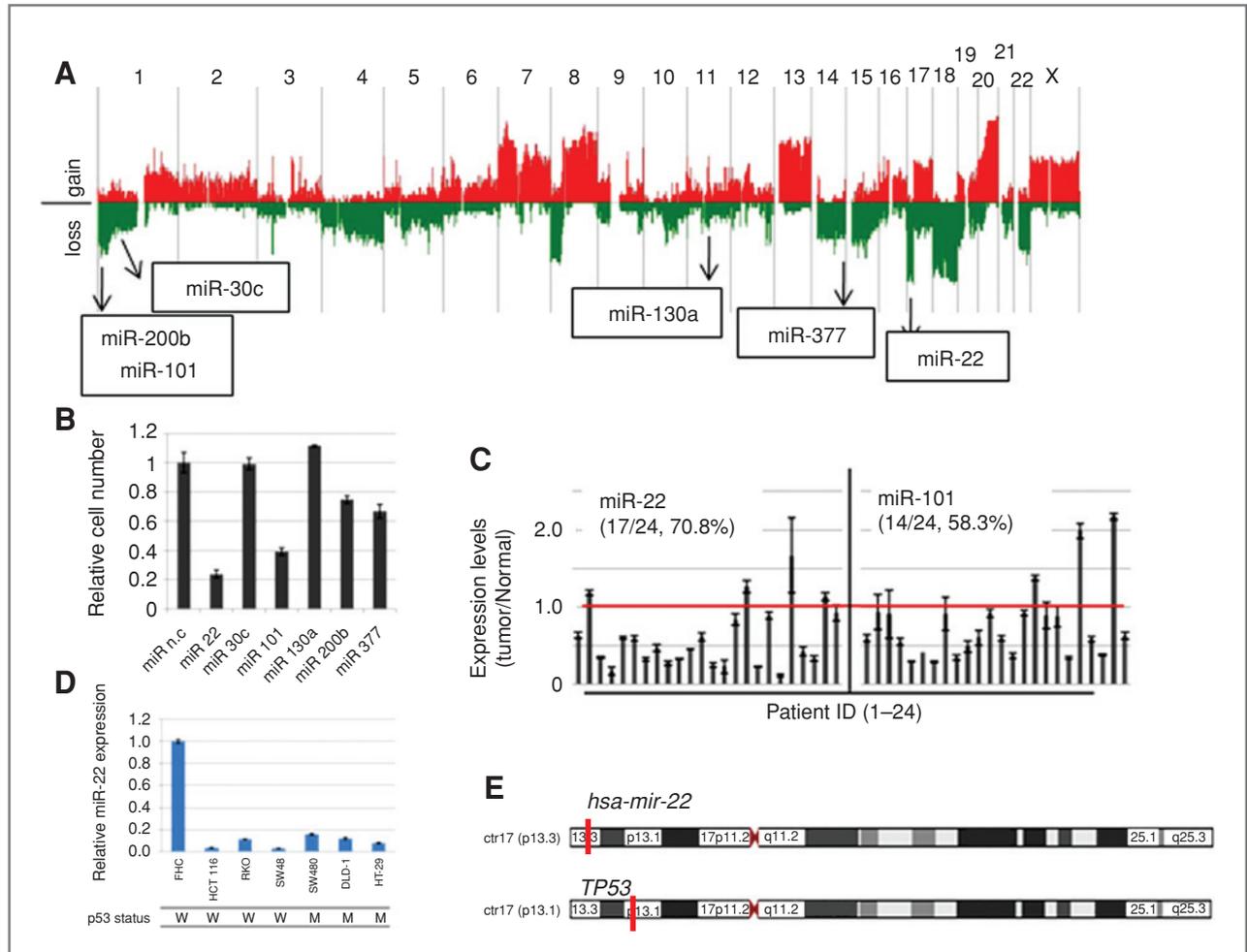


Figure 1. A, result of copy number aberrations in 24 human colon cancer samples. Red and green indicate chromosomal gain and loss, respectively. Chromosomal positions of 6 identified miRNA genes are shown in the CGH result. B, cell proliferation assay. HCT 116 cells were transfected with each synthetic miRNA and incubated for 5 days. Cell viability was measured by MST assay. Error bars indicate SD in triplicate cultures. C, expression of *miR-22* and *miR-101* in human colon cancer patients. Expression levels of *miR-22* and *miR-101* were quantified by TaqMan microRNA qRT-PCR. The graphs show the relative expression levels of *miR-22* and *miR-101*, calculated by adjusting their expression levels to matched normal counterparts in each cancer sample. The red line indicates the relative expression level of 1.0. D, expression of *miR-22* in human colon cancer cell lines and normal colon-derived FHC cells. The genomic status of *TP53* in cancer cell lines is indicated. E, chromosomal positions of *miR-22* and *TP53* genes on chromosome 17.

Table 1. Genomic status of *hsa-miR-22* and *TP53* genes in 24 human colon cancers

Sample No	Patient ID	has-miR-22 (17p13.3)		TP53(17p13.1)					
		chr17:1,563,947-1,564,081		Chr17:7,520,037-7,531,588					
		CNA	Expression (T/N)	CNA	Mutation	Exon	Codon	WT (A.A.)	Mut (A.A.)
1	1002	—	0.635	—	—				
2	1004	—	1.184	—	missense	7	237	ATG (M)	ATA (I)
3	1008	Loss	0.587	Loss	missense	6	193	CAT (H)	CGT(R)
4	1010	Loss	0.172	Loss	missense	8	285	GAG (E)	AAG (K)
5	1011	—	0.602	—	—				
6	1013	Loss	0.001	Loss	—				
7	1014	Loss	0.321	Loss	missense	7	230	GAA (E)	AAA (K)
8	1015	Gain	0.470	Gain	—				
9	1016	Loss	0.272	Loss	insertion (4)	7	280		
10	1017	Loss	0.327	—	—				
11	1018	Loss	0.455	—	—				
12	1019	—	0.614	—	—				
13	1022	Loss	0.248	Loss	missense	7	245	GGC (G)	TGC (C)
14	1023	Loss	0.227	Loss	missense	7	245	GGC (G)	TGC (C)
15	1024	Loss	0.835	Loss	missense	7	248	CGG (R)	CAG (Q)
16	1025	Loss	1.285	Loss	missense	5	175	CGC (R)	CAC (H)
17	1027	Loss	0.227	Loss	missense	7	248	CGG (R)	CAG (Q)
18	1028	Loss	0.888	Loss	missense	5	158	CGC (R)	CAC (H)
19	1029	Loss	0.114	Loss	—				
20	1033	Loss	1.668	Loss	deletion (1)	8	267		
21	1035	Loss	0.429	Loss	deletion (18)	5	174		
22	1036	Loss	0.339	Loss	missense	5	152	CCG (P)	CTG (L)
23	1037	Loss	1.139	Loss	—				
24	1039	Loss	0.922	Loss	deletion (6)	7	235	AAC (N)	ATG (M)

Two patients, sample numbers 10 and 11, showed hemizygous loss of the *miR-22* gene locus with intact *TP53*. Three patients, sample numbers 1, 5, and 12, showed downregulation of *miR-22* with intact *TP53*.

cancer (Fig. 1A and Supplementary Fig. S1E). Two of them, *miR-22* and *miR-101*, showed strong inhibition of cell proliferation in HCT 116-p53^{+/+} cells by MST assay (Fig. 1B). As shown in Fig. 1C, *miR-22* and *miR-101* showed reduced expression in 70.8% and 50.3% of colon cancer cases, respectively, when compared with their normal counterparts. *MiR-22* also showed significant downregulation in 6 colon cancer cell lines in comparison with FHC cells derived from normal colon epithelium (Fig. 1D), which was not observed for *miR-101* (data not shown). Interestingly, CGH analysis showed deletion of the *miR-22* locus without loss or mutation of *TP53* localized to the 6Mb centromeric region of the *miR-22* gene in 2 colon cancer patients, and 3 other cases showed a significant reduction of *miR-22* expression (Fig. 1E and Table 1). Furthermore, in a copy number assay using another set of colon cancer samples, 5 of 36 cases showed hemizygous

deletion of *miR-22* locus with intact copy of *TP53* (Supplementary Fig. S2).

Induction of apoptosis by *miR-22* in p53 wild-type colon cancer cells

Cell proliferation assays using the HCT 116-p53^{+/+}, HCT 116-p53^{-/-}, and p53 mutant SW480 cell lines showed a significant repression of cell proliferation by *miR-22* in 3 cell lines (Supplementary Fig. S3A). Interestingly, *miR-22* induced apoptosis selectively in HCT 116-p53^{+/+} cells (Fig. 2A and B). In contrast, it caused cell-cycle arrest in HCT 116-p53^{-/-} and SW480 cells (Supplementary Fig. S3B and C). These results indicate that *miR-22* acts as a growth repressor in colon cancer cells, and that its ability to induce apoptosis depends on the *TP53* status. Indeed, the expression profile of HCT 116 cells in the presence of *miR-22* showed significant modulation of

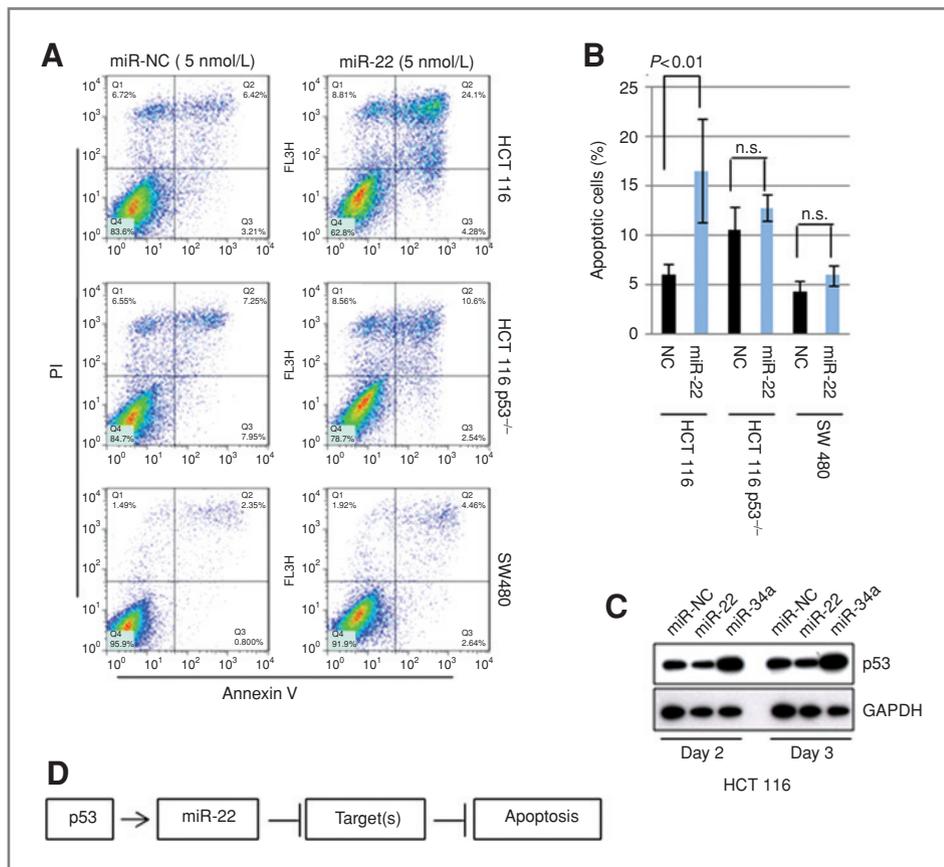


Figure 2. A, fluorescence-activated cell sorting (FACS) analysis. HCT 116, HCT 116-p53^{-/-}, and SW480 were transfected with 5 nmol/L of *miR-22* or *miR-NC*, incubated for 3 days, and subjected to FACS analysis. B, quantification of apoptotic cells. Apoptotic cells were quantified by using 4 independent FACS experiments. Data indicate the mean value with SD. Statistical analysis was carried out by *t* test. C, p53 is not activated by *miR-22*. Cells, transfected with *miR-22* or *miR-NC*, were incubated for 2 or 3 days, and subjected to immunoblotting. D, hypothesis of *miR-22* function in the p53 network.

cellular p53 network (Supplementary Fig. S3D and Tables S3 and S4). Furthermore, the introduction of *miR-22* into HCT 116 cells did not show upregulation or stabilization of p53, suggesting that *miR-22* may function downstream of the p53-induced apoptotic pathways, and that its role in the induction of apoptosis could be mediated by the repression of p53 target genes (Fig. 2C and D).

Identification of the *miR-22* gene as a direct transcriptional target of p53

As shown in Fig. 3A, *miR-22* is encoded within exon 3 of the *C17orf91* gene, which is located on the minus strand of the 17p13.3 region of the human chromosome, and consensus sequence of p53 binding sites (p53BS) was identified at a 5' upstream region and within the intron 2 of the *C17orf91* gene (Fig. 3A and Supplementary Fig. S4). The expression of *miR-22* was assessed in HCT 116-p53^{+/+} cells treated with 100 ng/mL of Adriamycin (ADR; doxorubicin, Bedford Laboratories), a genotoxic agent leading to activation of p53, for 24 hours. The result indicated that mature *miR-22* was increased considerably by ADR treatment in HCT 116-p53^{+/+} cells, but not in HCT 116-p53^{-/-} cells (Fig. 3B). The expression of *C17orf91* was induced only in HCT 116-p53^{+/+} cells by ADR (Fig. 3C). Transcriptional activation of *miR-22* was also found in HCT 116 cells after treatment with 5-FU, which was confirmed by qRT-PCR and

reporter gene analyses (Supplementary Fig. S5A–C). Furthermore, introduction of a cDNA encoding *C17orf91*, cloned by using a gene-specific primer set (Supplementary Fig. S4), into cells clearly showed an increase of mature *miR-22* in both p53 wild-type and p53^{-/-} HCT 116 cells (Fig. 3D). These results suggest that *miR-22* expression is regulated by p53 at the transcriptional level, not by p53-dependent processing during the maturation of the miRNA (32). Indeed, p53 binding on p53BS located at 5' upstream and intron 2 of the *miR-22* gene was significantly enhanced after exposure to 5-FU evidenced by p53 ChIP (Supplementary Fig. S5D and E). Furthermore, this was also confirmed by ChIP-sequencing (ChIP-Seq) analysis (Fig. 3E), indicating that *miR-22* is a direct transcriptional target of p53. The concurrent increase in tri-methylation of lysine 4 of histone H3 (33) evidenced transcriptional activation of the *miR-22* gene after exposure to 5-FU (Fig. 3E).

Identification of *p21* as a direct target of *miR-22*

To identify the *miR-22* target mRNAs involved in p53-dependent apoptosis, AGO2-immunoprecipitation (AGO2-IP) on ChIP analysis (31) was applied to screen mRNA species enriched in the AGO2 complex in a *miR-22* dependent manner; an *in silico* database search was further carried out using candidate mRNAs. This strategy was expected to lead to the efficient identification of responsible miRNA

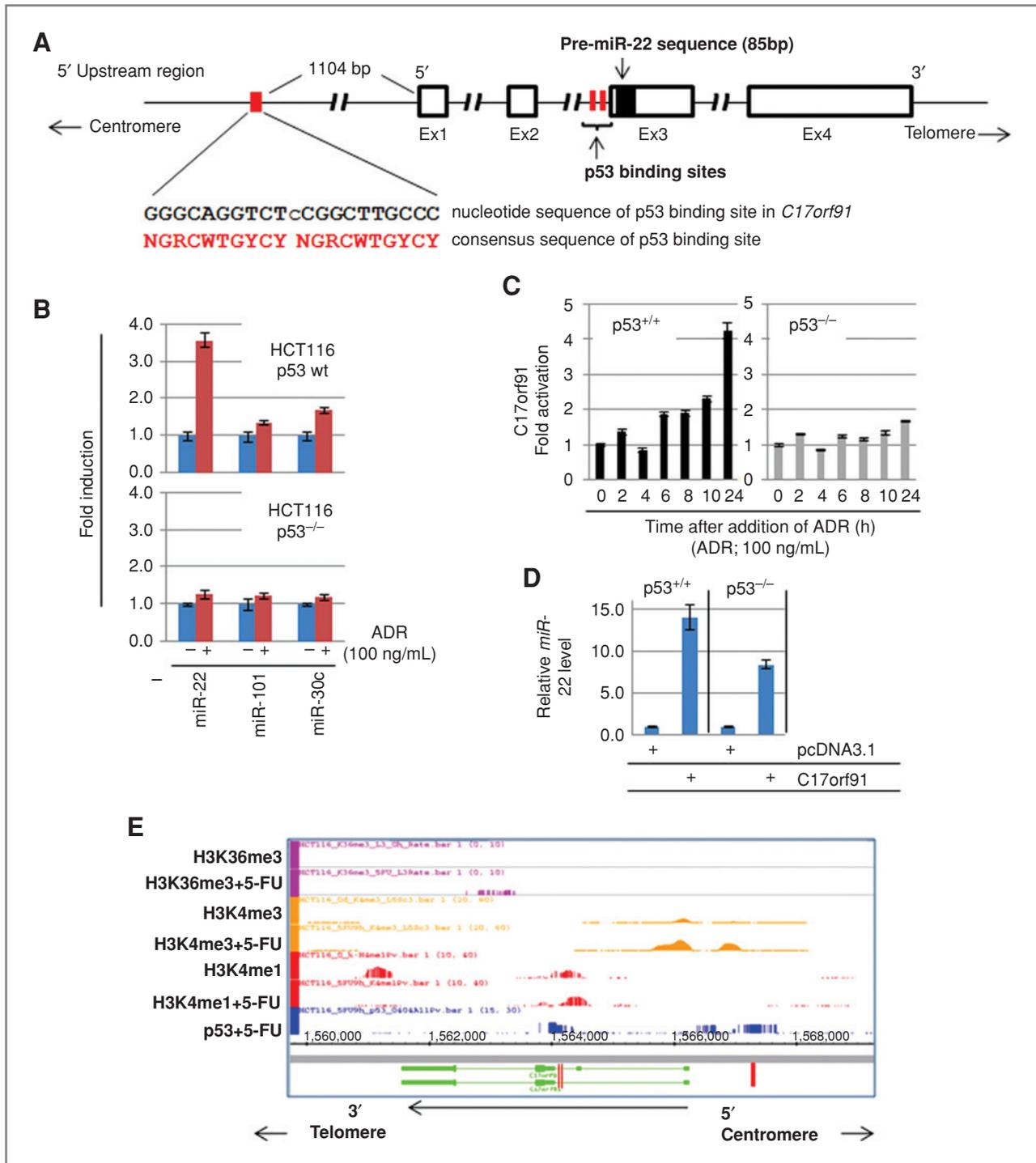


Figure 3. A, genomic structure of *miR-22* and its host gene, *C17orf91*. Genomic structure of *C17orf91* is indicated. Open boxes show exons and the region-encoded pre-*miR-22* is indicated by a closed box. Red boxes are p53 binding site at 5' upstream region and within intron 2. Consensus sequences of p53 binding sites located 5' upstream of exon1 of *C17orf91* are shown. B, induction of *miR-22* expression after addition of the genotoxic agent ADR in p53-wild type (wt) and p53^{-/-} HCT 116 cells. Cells were cultured in the presence or absence of ADR (100 ng/mL) for 24 hours. Mature-type miRNAs were measured by qRT-PCR. *miR-101* and *miR-30c*, whose expression was not affected by p53, were used as negative controls. C, upregulation of *C17orf91* by ADR. The cells were treated with ADR (100 ng/mL) for the indicated times, and *C17orf91* was quantified by TaqMan qRT-PCR. D, upregulation of *miR-22* by introduction of *C17orf91* cDNA. Cells were transfected with an expression vector containing *C17orf91* cDNA (Supplementary Fig. S4) for 48 hours. The expression of *miR-22* was analyzed by qRT-PCR. E, ChIP-sequence analysis. Genomic region of *C17orf91* indicates opposite direction as shown in (A) because of *C17orf91* gene encoded on minus strand in the chromosome 17. HCT 116 cells were treated with a DNA-damaging agent, 5-FU, for 9 hours, and ChIP was carried out by using the indicated antibodies. Red boxes show p53BS located at 5' upstream region and within intron 2. The direction of *C17orf91* gene is indicated by arrows.

targets. HCT 116 cells, stably expressing HA-AGO2, were transfected with *miR-22*, and the AGO2 complex was precipitated with anti-HA antibody, followed by the microarray analysis of the precipitated RNAs (Supplementary Fig. S6A). After calculation of the enrichment score (Supplementary Fig. S6B), 10 mRNAs were selected as *miR-22*-dependent AGO2-bound mRNAs, which included regulators of apoptosis and the cell cycle (Supplementary Fig. S6C and D). A search of the TargetScan database (34) using the top10 mRNAs revealed that only *p21* was a potential target for *miR-22*. Indeed, *p21* had a potential *miR-22* target sequence, whose site was conserved among the other mammalian species (Fig. 4A and Supplementary Fig. S6E). The expression of a luciferase reporter gene fused with the 3' UTR of *p21* mRNA was suppressed by the introduction of *miR-22* (Fig. 4B). This suppression was significantly reduced by the introduction of mutations into the *miR-22* response sequence (Fig. 4B, Mut1 and Mut2), indicating that *miR-22* represses *p21* directly. Furthermore, ectopic expression of *miR-22* in HCT 116-p53^{+/+} cells reduced p21 protein levels (Fig. 4C). Suppression of *p21* mRNA levels was also observed by introduction of *miR-22* (Fig. 4D). These results show that *miR-22* controls *p21* expression by both inhibition of translation and degradation of mRNA.

As shown in Fig. 4E, *miR-22* inhibited the ADR-induced upregulation of p21. Immunocytochemical analysis showed no nuclear accumulation of p21 in *miR-22* introduced cells, even after a 10-hour ADR treatment (Fig. 4F). To show that this repression occurs at a post-transcriptional, but not at a transcriptional level, the ADR-induced increase in *p21* mRNA was quantitatively assessed in the presence or absence of *miR-22*. As expected, transcriptional activation of *p21* was observed with similar kinetics as the p53 response in both miR-NC and *miR-22* introduced cells after ADR treatment (Fig. 4G). These observations suggest that *miR-22* directly represses *p21* expression via a post-transcriptional mechanism.

Sensitization of p53-dependent apoptosis by *miR-22*

p21 is known to be a key regulator of cell-cycle arrest after the activation of p53, and also an inhibitor of apoptosis (35). Thus, we analyzed the effect of *miR-22* levels on the p53-dependent apoptosis. HCT 116-p53^{+/+} cells were transfected with either *miR-22* or miR-NC, and apoptotic cells were quantified by FACS in the presence or absence of ADR. As shown in Fig. 5A, cells transfected with miR-NC showed a slight increase of the Annexin V and PI double-positive fraction after 12-hour exposure to 100 ng/mL of ADR (Fig. 5A, top right and B). The introduction of low amounts (2 nmol/L) of *miR-22* slightly enhanced the induction of apoptosis compared with those with miR-NC in the absence of ADR (Fig. 5A, bottom left, and B). The addition of ADR caused a marked increase of apoptotic cells in *miR-22*-transfected cells (Fig. 5A, bottom right, and B), indicating that *miR-22* sensitizes cells to p53-dependent apoptosis induced by DNA damage. Next, we analyzed the effect of p21 protein levels on *miR-22*-induced apoptosis. *miR-22* caused significant repression of p21 upregulation by ADR treatment for 24 hours (Fig. 5C, lanes 2 and 5,

and Supplementary Fig. S7A). The introduction of p21 ORF showed the reduction of apoptosis, evidenced by the decrease in PARP-1 cleavage (36), in cells transfected with *miR-22* (Fig. 5C, lanes 5 and 11). This was reproducibly detected (Supplementary Fig. S7B).

These results suggest that endogenous levels of *miR-22* are a cellular determinant for the induction of apoptosis through the repression of p21. On the other hand, p21 knockdown induced the cleavage of PARP-1 (Supplementary Fig. S7C). This was consistent with previous reports that p21 deficiency sensitizes cells to apoptosis (37, 38). However, p21 knockdown was not as prominent as is observed by *miR-22* introduction. This strongly suggests that other factors, being also regulated by *miR-22*, could be involved in the sensitization of p53-dependent apoptosis by *miR-22*. Furthermore, inhibition of *miR-22* by expression of an antisense *miR-22* transcript causes the substantial decrease of S-phase cells, suggesting the cell-cycle arrest at G₁ phase (Supplementary Fig. S7D).

Transcriptional activation of *miR-22* depending on the intensity of stresses

To examine whether the expression of *miR-22* and *p21* levels correlate with the induction of apoptosis in a physiologic setting, the kinetics of *miR-22* and *p21* mRNA expression was examined by treating cells with different doses of ADR. As expected, HCT 116 cells treated with 50 ng/mL of ADR showed cell-cycle arrest, but no apoptosis, with rapid increments of *p21* at both mRNA and protein levels; upregulation of *miR-22* was not observed, even after the ADR-mediated activation of p53 (Fig. 6A, left graph, and 6B, left). Under a high-dose exposure to ADR, in contrast, the expression levels of *p21* mRNA and *miR-22* increased from 8 hours after the addition of 200 ng/mL of ADR (Fig. 6A, right). Interestingly, p21 protein levels were not elevated significantly after 36 hours of incubation with ADR, despite the striking increase in *p21* mRNA level (Fig. 6B, top right). The PARP-1 cleavage was observed at a similar kinetics with *miR-22* expression (Fig. 6B). Similarly, a significant activation of *miR-22* accompanying the repression of p21 protein and increase of PARP-1 cleavage was also observed in HCT 116 cells after exposure to high doses of actinomycin D (Act D), an inhibitor of RNA polymerases that activates p53 (ref. 39; Fig. 6C and D). ChIP analysis indicated the enhancement of p53 binding to p53BS in the *miR-22* gene only after addition of high doses of Act D (Supplementary Fig. S8A and B). Interestingly, treatment with deferoxamine, an inducer of HIF1 α that stabilizes and activates p53 (40), did not upregulate *miR-22* or *p21* mRNA and did not induce apoptosis despite the activation of p53 (Fig. 6C and D). These results indicate that the activation of *miR-22* regulated by p53 is dependent on the strength and type of stresses.

Discussion

In the present study, *miR-22* was identified as a strong candidate for tumor suppressor gene in human colon cancers,

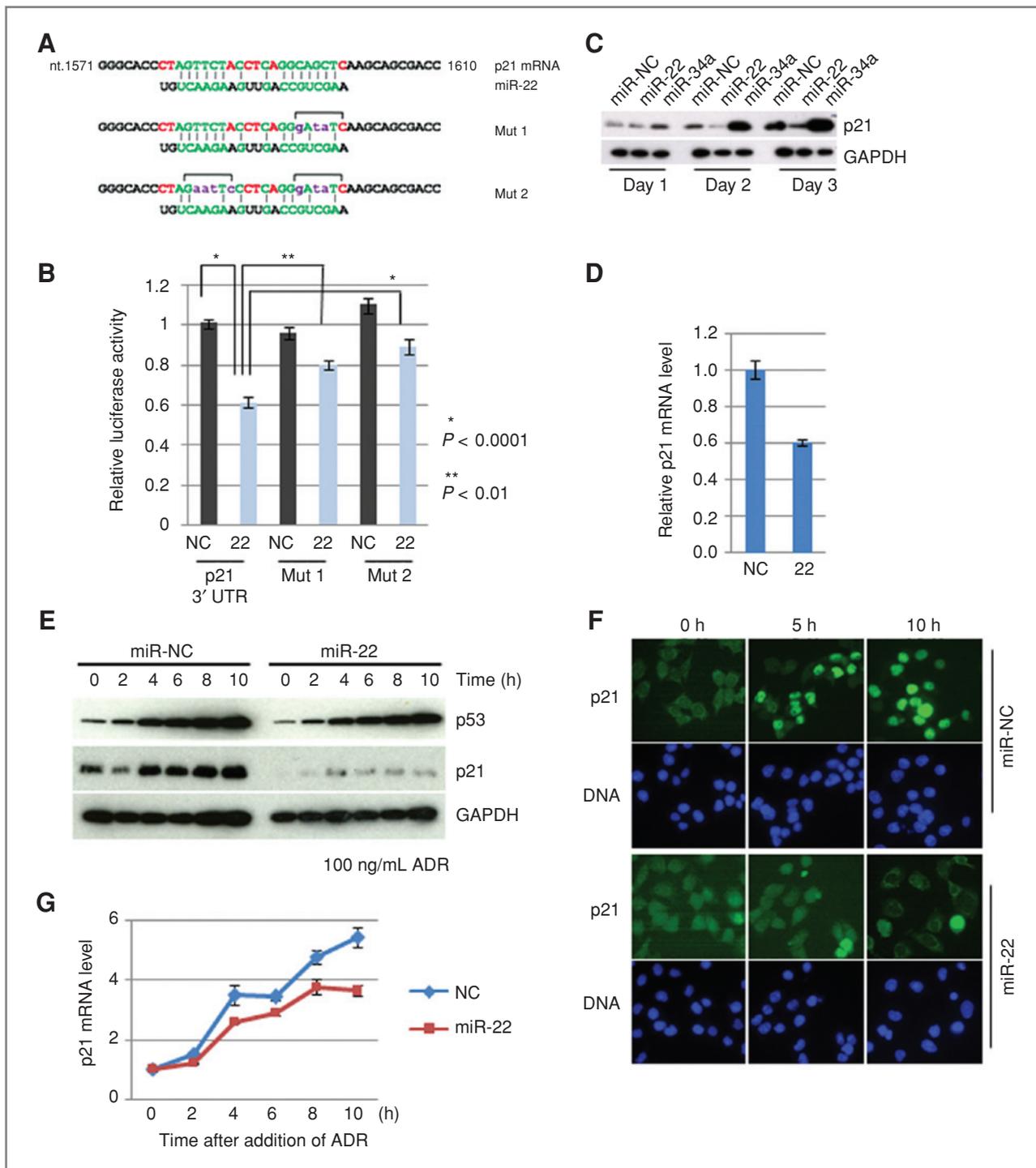


Figure 4. A, sequence alignment of *miR-22* and the 3' UTR of *p21* mRNA is indicated at the top. Mutant sequences used for the reporter gene assay are listed (Mut 1 and Mut 2). B, reporter gene assay. Error bar indicates SD ($n = 6$). C, expression level of p21 protein in the presence of *miR-22*. *miR-34a* was used as positive control. D, expression levels of *p21* mRNA in the presence of *miR-22*. Cells were transfected with *miR-22*, and incubated for 3 days. The relative expression levels of *p21* mRNA were quantified by TaqMan assay. E, effect of *miR-22* on the activation of p21 expression after exposure to ADR. HCT 116 cells were transfected with 5 nmol/L of either *miR-22* or *miR-NC* and incubated for 48 hours, and further incubated in the presence of ADR for the indicated times. F, indirect immunocytochemistry. Cells were transfected as described above, and incubated in the presence of ADR for the indicated times. Cells were subjected to immunostaining. G, activation of *p21* expression in the presence or absence of *miR-22* after exposure to ADR. Cells were prepared as described in (E), and total RNAs were prepared from each time point. Relative expression levels of *p21* mRNA were quantified by TaqMan assay.

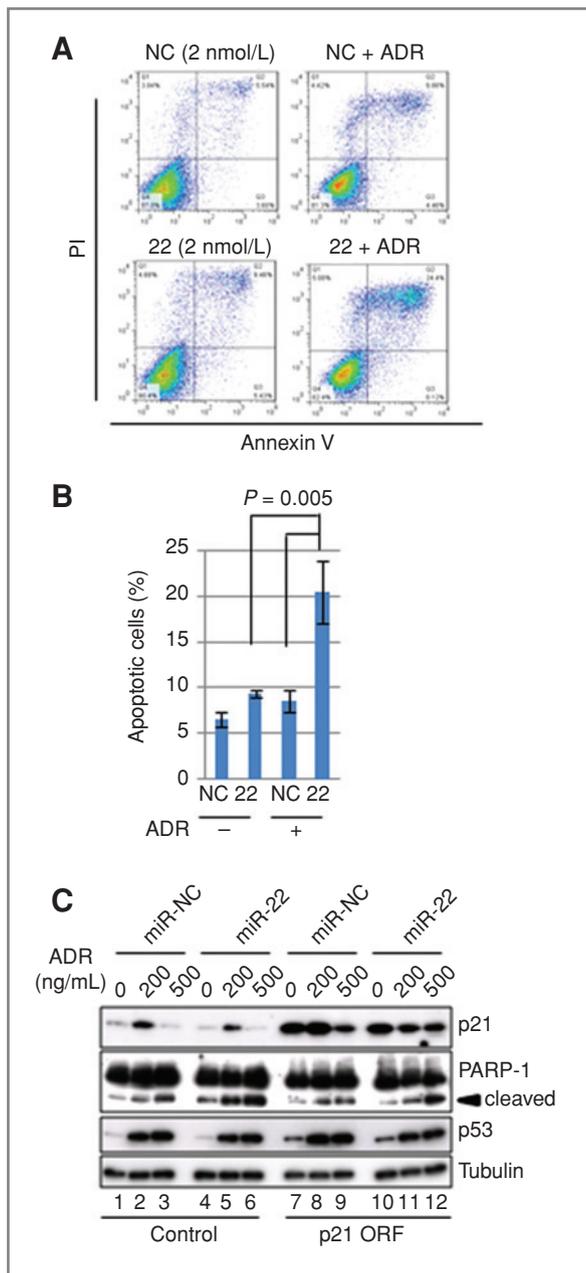


Figure 5. A, sensitization to p53-dependent apoptosis by *miR-22*. HCT 116 cells, transfected with either 2 nmol/L of *miR-22* or miR-NC, were incubated in the presence or absence of 100 ng/mL ADR for 12 hours. Apoptotic cells were determined by FACS. B, quantification of apoptotic cells using 3 independent FACS experiments as described in (A). Data show mean with SD. C, expression of p21 protein reduced *miR-22*-induced sensitization of apoptosis. HCT 116 cells were transfected with either control or p21 ORF lentivirus. After selection, cells transfected with either miR-NC or *miR-22* were treated with indicated concentration of ADR for 24 hours and cleaved PARP-1 was detected by immunoblotting.

and its role in the determination of p53-dependent cellular fate through the formation of the p53-*miR-22*-p21 axis was shown. This axis might be activated by specific stresses that require the elimination of damaged cells. The current

findings provide a novel insight into the regulatory mechanism of cell fate determination by a specific molecule, *miR-22*, in response to various oncogenic stresses and in a p53-dependent manner.

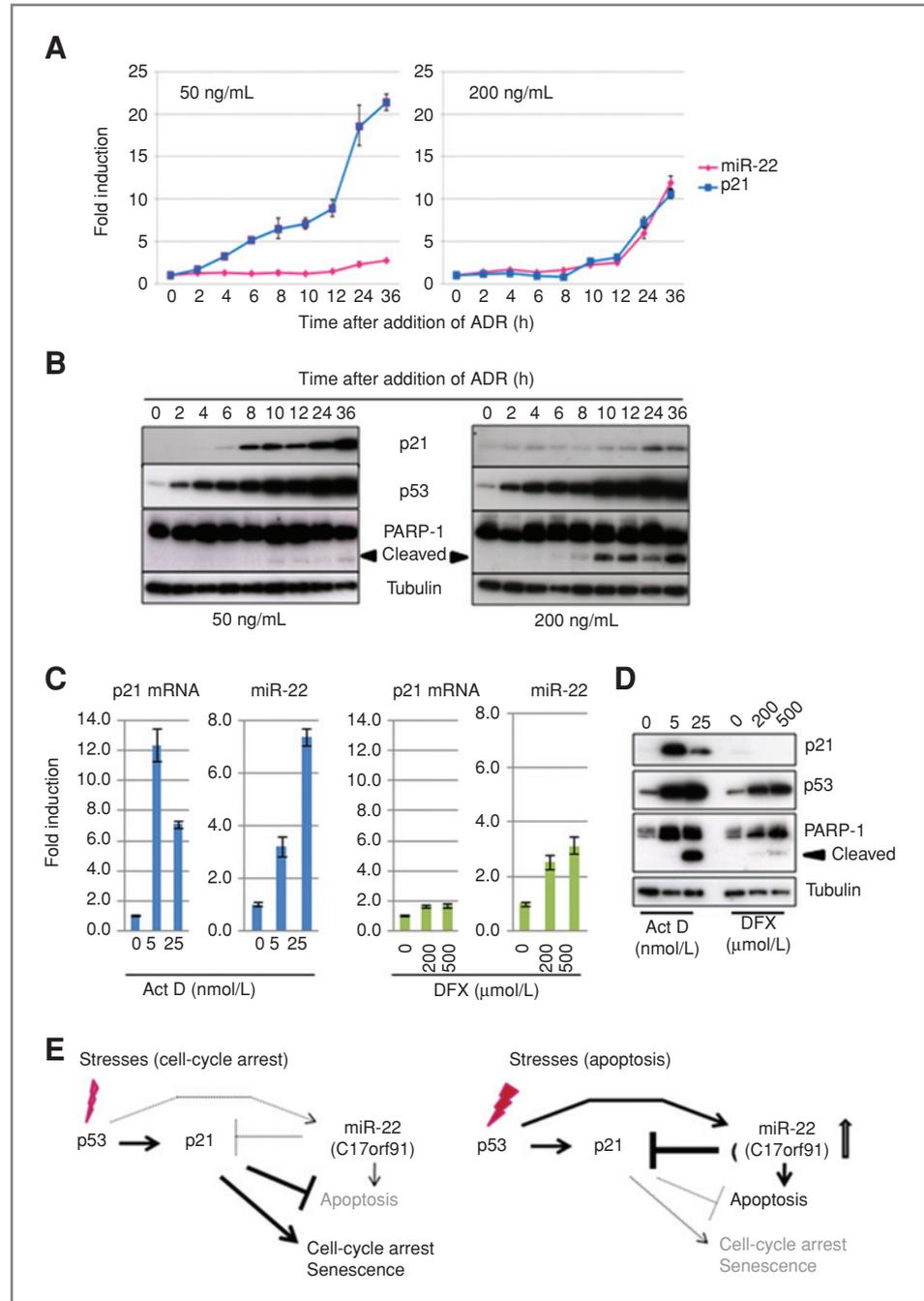
As depicted in Fig. 6E, 2 modes of action of the p53-*miR-22*-p21 axis were suggested in response to the different intensity of the stresses applied. In brief, p53 only activates p21 to induce cell-cycle arrest against weak stresses in the p53-p21 pathway. On the other hand, severe damage transcriptionally activates both p21 and *miR-22*, and *miR-22* represses p21 expression through the inhibition of protein synthesis and enhancement of p21 mRNA degradation. Under severe damage conditions, apoptosis may be induced by entry into the cell cycle via direct repression of p21 by *miR-22*.

Antiapoptotic function of p21 has recently attracted attention for its oncogenic action, which is opposed to a traditional tumor suppressor function. Lack of p21 induces apoptosis through the accumulation of DNA damage in leukemic stem cells (41). Disruption of the p21 gene sensitized cancer cells to apoptosis after treatment with chemotherapeutic agents (37, 38). Recently, the small molecule RITA, an activator of p53, was shown to efficiently induce apoptosis through inhibition of p21 (42). Furthermore, a single recombinant adenovirus containing p53 cDNA and synthetic p21 shRNA also efficiently induced apoptosis in colon cancer cell lines (43). These findings indicate that the downregulation or inhibition of p21 after activation of p53 in stressed cells is one of the key factors as an anticancer mechanism by inducing the change of cellular phenotype from cell-cycle arrest to apoptosis, which could be the mechanism triggered by *miR-22* as an intrinsic stress-response network.

MicroRNAs are known to repress multiple target mRNAs, leading to efficient shut down or activation of intracellular networks (44, 45). Indeed, introduction of *miR-22* broadly and significantly modulates cellular networks in p53 wild-type HCT 116 cells (Supplementary Tables S3, S4, and Fig. S3D). Furthermore, high levels of expression of *miR-22* alone clearly showed apoptosis without activation of p53 in HCT 116 cells (Fig. 2A), where p21 might not be a promising target of *miR-22*, suggesting that other *miR-22* target genes also contribute to the induction of p53-dependent apoptosis.

In addition to the *miR-22* function in p53 wild-type colon cancer cells, another interesting feature is that *miR-22* expression induces cell-cycle arrest in p53 knock-out and mutant cell lines (Supplementary Fig. S3B and C). We searched for a TargetScan database to obtain a list of potential *miR-22* targets and conducted gene ontology analysis to identify genes whose repression theoretically induces cell-cycle arrest. These analyses indicated that several positive cell-cycle regulators, *CDK6*, *CDK3*, *SIRT1*, *CDC25B*, and *HDAC4*, are possible targets of *miR-22*. We analyzed the protein levels of CDK6 and SIRT1 by immunoblot analysis, and found no significant changes in their protein levels in the presence of *miR-22* in SW480 cells. Then, we re-evaluated the data of AGO2-IP on ChIP analysis using HCT 116 cells. Interestingly, *CDK3*, *CDC25B*, and *HDAC4* mRNAs were enriched in the AGO2 complex in a *miR-22*-dependent manner (data not shown). Although it is currently unclear that these mRNAs are directly

Figure 6. A and B, kinetics of *miR-22* and *p21* increments after exposure to ADR. Time-dependent increments of *p21* mRNA and *miR-22* after exposure to different doses of ADR were quantified by RT-PCR. Relative expression of *p21* and *miR-22* was calculated by $2^{-\Delta\Delta ct}$ using *GAPDH* as an internal standard (A). Protein levels of *p21*, *p53*, and cleaved PARP-1 were analyzed by immunoblotting in cells treated with different doses of ADR (B). C and D, activation of *miR-22* expression by Act D. Cells were treated with specific concentration of either Act D or deferoxiamine for 24 hours, and *p21* and *miR-22* levels were determined as described above. E, 2 modes of action of *miR-22* in the p53 network.



downregulated by *miR-22*, *miR-22* may induce p53 independent cell-cycle arrest through the repression of these genes.

The present data suggest a tumor-suppressive role of *miR-22* in colon cancer cells. *miR-22* was also reported to be downregulated in estrogen receptor (ER)-positive breast cancers, and repression of ER expression by *miR-22* suppressed cell proliferation (46, 47). On the other hand, *miR-22* was recently suggested to have an oncogenic role through the direct silencing of *PTEN* and its upregulation in prostate cancer cell lines (48). These authors identified the

transforming activity of *miR-22* in mouse embryonic fibroblast cooperatively with *c-Myc*, and showed that the overexpression of *miR-22* in the prostate cancer cell line DU145, harboring p53 mutations in both alleles, caused an enhancement of colony formation. The reported paradoxical function of *miR-22* implies that *miR-22* could act as a tissue-specific or context-dependent tumor suppressor gene.

Chromosome 17p13.3, where the *miR-22* gene resides, is well known to be a target for allelic loss, and loss of heterozygosity in 17p13.3 is often found independently of

the *TP53* mutation in human cancers, including lung and breast cancer (49, 50). Furthermore, an unknown tumor suppressor gene has been suggested to be present at this locus. The present data suggest that *miR-22* is a candidate haploinsufficient-type tumor suppressor gene within this region, and its hemizygous loss or downregulation reduced apoptosis induction in response to stresses, even in cells retaining an intact *TP53*.

In summary, the data presented suggest a role for *miR-22* as an intrinsic molecular switch in the p53 tumor suppressor network, functioning as a determinant of cell fate at a post-transcriptional level by inducing apoptosis via direct repression of *p21*. This system might function in the p53-dependent activation of a specific anticancer barrier in response to various oncogenic stresses, and dysfunction of *miR-22* might confer a chance of survival for damaged cells with tumorigenic potential.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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